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Translating molecular advances in Down syndrome and Fragile X syndrome into therapies.

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## Abstract

Ongoing treatments for genetic developmental disorders of the central nervous system are mostly symptomatic and do not correct the genetic cause. Recent identification of common mechanisms between diseases has suggested that new therapeutic targets could be applied across intellectual disabilities with potential disease-modifying properties. The European Down syndrome and other genetic developmental disorders (DSG2D) network joined basic and clinical scientists to foster this research and carry out clinical trials. Here we discuss common mechanisms between several intellectual disabilities from genetic origin including Down's and Fragile X syndromes: i) how to model these complex diseases using neuronal cells and brain organoids derived from induced pluripotent stem cells; ii) how to integrate genomic, proteomic and interactome data to help defining common mechanisms and boundaries between diseases; iii) how to target common pathways for designing clinical trials and assessing their efficacy; iv) how to bring new neuro-therapies, such as noninvasive brain stimulations and cognitive training to clinical research. The basic and translational research efforts of the last years have utterly transformed our understanding of the molecular pathology of these diseases but much is left to be done to bring them to newborn babies and children to improve their quality of life.

### **Common mechanisms causing intellectual disabilities**

Down syndrome (DS) and Fragile X syndrome (FXS) are the most common genetic causes of intellectual disability (ID), for which no approved therapies are available yet. These disorders are associated with neurological complications including cognitive deficits that lead to mild to profound impairment in intellectual functioning. Current therapeutic approaches focus on behavioral therapy, educational mainstreaming and off-label medications that mitigate only a limited set of symptoms, such as hyperactivity, some cognitive deficits, seizure and anxiety. Individuals with DS, mostly males and approximately 30% of females affected by FXS, have significant intellectual deficits and social dysfunctions in adulthood, with deleterious impact on affected individuals, families and society (Lott & Dierssen, 2010; Maurin *et al.*, 2014).

DS and FXS show striking similarities and differences. Both intellectual disabilities are genetic developmental disorders characterized by defects in structural and synaptic plasticity due to alterations in specific molecular pathways leading to cognitive impairment. Interestingly, neuroanatomical abnormalities are probably generated early during development in the brain of patients and in mouse models for DS and FXS, and may be associated to defects in proliferation and/or differentiation of neural progenitors (Guidi *et al.*, 2014; Castren, 2016), pointing out a critical role of Fragile X Mental retardation Protein (FMRP) and human chromosome 21 genes during neurogenesis. Children with DS (aged between 5 and 23 years) have smaller overall brain volumes with smaller cerebellum and larger subcortical grey matter volumes (Pinter *et al.*, 2001), while children with FXS (aged between 18 and 42 months) have larger brain volumes and display enlargement in the temporal lobe white matter, cerebellar gray matter and caudate nucleus, but have a smaller amygdala (Hazlett *et al.*, 2012). In a study analyzing boys with FXS aged 1 to 3 years, the authors found, as in the adult patients, increased caudate, fusiform gyrus, and thalamus grey matter volume (GMV) as well as reduced GMV in the superior temporal gyrus, hippocampus, insula, hypothalamus, and orbitofrontal cortex, and medial and lateral prefrontal cortices (Hoeft *et al.*, 2010).

At the cellular level, children and adults with DS show dendritic atrophy with a significant decrease in dendritic branching, length and spine density (Takashima *et al.*, 1989). FXS patients exhibit increased density of immature dendritic spines that appear longer, denser and thinner (Irwin *et al.*, 2000a; Irwin *et al.*, 2000b). Similarly, pyramidal cells from mouse model of DS differs significantly in their dendritic arborization and branching pattern, which is also accompanied by immature dendritic spines (Dierssen *et al.*, 2003). In addition, DS patients show reduced dendritic branching and complexity in pyramidal neurons along with fewer and abnormal spines with enlarged heads that could explain the cognitive deficits (see Dierssen, 2012 for review). This goes along with alterations in synaptic plasticity molecular pathways leading to long-term potentiation (LTP) deficits in DS mouse models, while long-term depression (LTD) is enhanced. Conversely, in FXS patients, while LTP is reduced in cortex, hippocampus and amygdala (Desai *et al.*, 2006; Lauterborn *et al.*, 2007; Suvrathan & Chattarji, 2011; Seese *et al.*, 2012), hippocampal mGluR-dependent LTD is strongly induced, due to the overactivation of metabotropic glutamate receptor 5 (mGluR5) (Huber *et al.*, 2002).

Those changes stem from very different genetic causes. DS is due to the presence of an extra copy of human chromosome 21 (HSA21) containing approximately 270 genes. While not all three copy genes are overexpressed in the DS conditions (Ait Yahya-Graison *et al.*, 2007) there are several candidate genes that are transcribed in the central nervous system and overexpressed in the DS condition, whose expression and/or biological activity could be reduced with therapeutic agents. Among them, the kinase Dual Specificity Tyrosine-(Y)-Phosphorylation Regulated Kinase 1 (DYRK1A), which is involved in brain development is closely associated to DS phenotype and can be modulated using different classes of inhibitors (Duchon & Herault, 2016). Several mouse models of DS were engineered that reproduce phenotypes common with DS including cognitive impairments (Choong *et al.*, 2015). Using these mouse models more than 50 therapies targeting neurotransmission,

neuroprotection or specific gene overexpression have been tested so far with a high rate of success (Stagni *et al.*, 2015).

FXS is due to the silencing of the Fragile X Mental Retardation gene 1 (*FMRI*) encoding the RNA binding protein FMRP endowed with multiple RNA binding domains, a Nuclear Localization Signal (NLS) and a Nuclear Export Signal (NES). This protein is mainly expressed in neurons and implicated in several steps of RNA metabolism including i) translational regulation, being part of ribonucleoproteic complexes associated to polyribosomes both in soma and at the synapse; ii) export of mRNA from nucleus to cytoplasm; and iii) transport along dendrites and axons. The mRNA targets of FMRP have been extensively studied. Even if functional characterization has been performed only for some of them, it is clear that FMRP is mostly involved in regulating the levels of synaptic proteins implicated in autism (Maurin *et al.*, 2014). A mouse model for FXS exists, recapitulating the human phenotype of the disorder. By using this mouse line, in recent years, the increased knowledge about the molecular pathways that are altered in FXS neurons has led to the identification of metabotropic glutamate receptor 5 (mGlu5) and GABA<sub>B</sub> receptors as potential pharmacological targets (Ellegood *et al.*, 2010; D'Antoni *et al.*, 2014; de Esch *et al.*, 2014; Lai *et al.*, 2016).

Among the intellectual disabilities from genetic origin, the chromosome 22q11.2 microdeletion is one of the strongest genetic risk factor for neurodevelopmental disorders, therefore an ideal genetic defect to study molecular pathways implicated in neurodevelopmental affections that result from imbalances in gene dosage. This microdeletion removes one copy of a chromosomal region spanning ~63 genes. This observation raises the question of whether there are single or diverse pathways downstream of this complex polygenic genetic defect and the identity of these pathways. To address these questions, the research group led by Victor Faundez developed a pedigree-based quantitative mass spectrometry strategy, *genealogical proteomics*, where they compared the whole proteome of probands affected by 22q11.2 microdeletion syndrome and early childhood psychosis to the

proteomes of their unaffected relatives (Zlatic *et al.*, 2018). They analyzed homogenates of primary cultured skin fibroblasts from five affected individuals and four families using Tandem-Mass-Tagging (TMT), triple Stable Isotope Labeling with amino acids in cell culture (SILAC), and Label Free Quantitative (LFQ) MS/MS, and used bioinformatics to infer molecular mechanisms co-segregating with the microdeletion. In this study ~1,200 proteins whose expression was modified by the 22q11.2 microdeletion were identified with expression changes significantly enriched in ontological categories of mitochondrion and actin filament cytoskeleton. Those changes were confirmed in a mouse model that genetically mimics the 22q11.2 microdeletion (*Df(16)A*) using similar proteomic and bioinformatics strategies. Focusing on the proteins expressed in mitochondria, Faundez and coll. confirmed that 22q11.2 mutant cells had distinctive respiration mitochondrial phenotypes and using *Drosophila* they demonstrated that dosage reductions of mitochondrial gene products identified in 22q11.2 cells affected synaptic transmission and behavior. These results demonstrate that several pathways are affected downstream the 22q11.2 mutation. One of these routes involves the mitochondria and its genetic manipulation affects synaptic function in *Drosophila*.

Altogether, these results shed light on common or divergent mechanisms that can give rise to similar or opposite phenotypes (Table 1). However, despite high hopes, preclinical studies have not always translated into a broadly effective treatment for intellectual disabilities of genetic origin.

### **Using induced pluripotent stem cells, derived neuronal cells and brain organoids to model DS and FXS**

As highlighted above, despite continuous efforts to improve the process of drug discovery, achieving success at the clinical stage remains challenging. During the last years, the discovery of human induced pluripotent stem cells (iPSC), a type of pluripotent stem cell that can be generated directly from adult cells, has opened up the doors to a new era of disease modeling and brought new opportunities to drug discovery field. For intellectual disabilities, availability of human iPS-derived



cells is enabling scientists to access a variety of human cellular models which can be used as tools to improve understanding of disease mechanisms and test therapeutic targets. However, their use in the field of translational medicine remains challenging and requires high levels of scrutiny and validation at each stage. Even though these models proved useful in investigating single gene disorders, it is not known yet how useful iPSCs will be in modeling complex human diseases, such as DS. Here we will discuss some of the most recent advances in this field.

Some years ago, an isogenic DS iPS cell integration-free model was developed in the group of Dean Nizetic by using fibroblasts of an adult with constitutional mosaicism for trisomy 21 (T21). This allowed comparing T21 and control isogenic neurons and recapitulating several DS phenotypes, such as increased  $\beta$ -amyloid, mitochondrial abnormalities, and increased DNA double strand breaks that indicated accelerated ageing (Murray *et al.*, 2015). A collection of iPSCs was generated from individuals with DS through the *LonDownS* consortium in London UK (<https://www.ucl.ac.uk/london-down-syndrome-consortium>). So far, >400 DS adults have been recruited with >120 lines isolated. Another interesting experimental material is the 3D cerebral organoids generated by culturing iPSCs in a three-dimensional rotational bioreactor over several months which recapitulate aspects of human brain structure and layering (Sutcliffe & Lancaster, 2017). These new human cellular models and organoids will be very valuable to evaluate pharmacotherapies for improving intellectual disabilities.

Also in the FXS field, murine embryonic stem cell lines derived from the inner cell mass of early mouse embryos contributing to all tissues including germline tissue and displaying a reduced expression of *Fmr1* by stable transfection of a specific shRNA directed against *Fmr1* (*shFmr1* ES) were generated by the group of Barbara Bardoni, providing a cellular model of FXS, as it shows FMRP depletion (Khalfallah *et al.*, 2017). These cells do not display any cell cycle variation or morphological abnormality but exhibit altered expression of a subset of genes mainly involved in

neuronal differentiation and maturation, suggesting a subjacent molecular pathology. For this reason, Bardoni and coll. induced differentiation of sh*Fmr1* ES cells into the neuronal lineage. In the protocol, after 4 days of *in vitro* differentiation, they detected an accelerated generation of neural progenitors and neurons with an increased expression of Cyclin-dependent kinase inhibitor 1B (p27<sup>Kip1</sup> or p27),  $\beta$ III-Tubulin. After one week of *in vitro* culture they observed a transient reduction of the number of nuclei, that did not affect the final number of neurons at late phases of *in vitro* neurogenesis (after 2-3 weeks of *in vitro* culture). Interestingly, neurogenesis was also accelerated in the embryonic brain of *Fmr1* KO mice, where they detected an elevated level of p27 and  $\beta$ III-Tubulin both at E12.5 and E14.5. These findings suggest that the sh*Fmr1* ES cell model recapitulates the molecular and cellular alterations present *in vivo* (Khalfallah *et al.*, 2017). The accelerated generation of neural progenitors and neurons during the first steps of neurogenesis of sh*Fmr1* ES cells is likely due to an elevated level of the Amyloid Precursor Protein (APP), whose mRNA is a known target of FMRP (Westmark & Malter, 2007). APP is processed by the  $\beta$ -site Amyloid precursor protein Cleaving Enzyme 1 (BACE-1), producing the  $\beta$ -amyloid (A $\beta$ ) peptide that is known to accelerate neurogenesis by activating the expression of *achaete-scute family bHLH transcription factor 1 (Ascl1)* (Uchida *et al.*, 2007; Freude *et al.*, 2011), a factor that has a pivotal role in neuronal differentiation. Thus, in *Fmr1*-depleted ES cells the elevated level of A $\beta$  peptide is likely to induce the expression of *Ascl1* that represents a surprising event and the key point to explain the subsequent accelerated neuronal differentiation (Khalfallah *et al.*, 2017). Consistently, the cell phenotype is rescued not only by re-expressing human FMRP, but also by reducing the processing of APP by the specific BACE-1 inhibitor LY2811376. The importance of the A $\beta$  peptide in the pathophysiology of FXS as well in other forms of autism and intellectual disabilities (ID) has been extensively studied (Westmark *et al.*, 2016).

The phenotype of sh*Fmr1* neural progenitors appears surprising since cell models of neural precursors for genes involved in other forms of ID/autism and DS rather display a delay of neuronal

differentiation or a disruption of neurogenesis (Jolly *et al.*, 2013; Jolly *et al.*, 2015; Fujitani *et al.*, 2016). An example of accelerated neuronal differentiation is provided by Alpha Thalassemia/mental Retardation syndrome X-linked (ATRX) intellectual disability. Indeed, premature neurogenesis goes along with gross brain abnormalities consistently with the microcephaly observed in patients affected by this disorder (Ritchie *et al.*, 2014; Huh *et al.*, 2016). Conversely, the depletion of Phosphatase and TENsin homolog (PTEN) in postnatal/young neural stem cells produced an altered neurogenesis characterized in a first step by an increased proliferation and differentiation rate of these cells (Amiri *et al.*, 2012) followed by an early loss of Neural Stem/progenitor Cells (NSCs). In this case, similarly to FXS, it is possible to observe an altered kinetics of neurogenesis. However, due to the severity of the cellular alterations, the morphological brain abnormality appears more evident than in FXS brains (Maurin *et al.*, 2014; Khalfallah *et al.*, 2017; Westmark, 2017).

The sh*Fmr1* cell model will be a very useful tool to search for novel therapies for FXS. Indeed, it can be used for screening of bioactive molecules, including libraries of small bio-active molecules approved for clinical use. This screening is feasible considering that we have shown that the phenotype of the FXS cell model can be reverted by pharmacological tools such as BACE1 inhibitors and some outputs can be easily measured even in a high-throughput screening format leading to the identification of new drugs to treat FXS (Bardoni *et al.*, 2017). Overall, these results underline the importance of studying embryonic neurogenesis in ID animal models to decipher the pathophysiology of these disorders and to identify biomarkers for translational studies.

### **Targeting common pathways altered in DS and other neurodevelopmental disorders**

During the past years, many groups intensively worked to clarify the molecular mechanisms underlying impaired brain functioning in DS, FXS and other developmental disorders and to devise targeted central nervous system therapies. Neurogenesis and dendritogenesis alterations are the two major defects of the DS and FXS brains and are present at the very beginning of brain development.

Thus, it is important to treat DS and FXS individuals during the perinatal period. Here we present a number of targeted hypothesis-driven therapies tested in different mouse models to identify drugs that eventually will be effective in clinical trials.

Lithium was one of the first to be tested since it was known that in adult mice it increases neurogenesis in the subventricular zone of the lateral ventricle (Bianchi *et al.*, 2010a). Lithium however, was not well tolerated by the pups that exhibited a very high death rate. In the following studies, fluoxetine, a selective serotonin reuptake inhibitor, was used because serotonin is crucial for neurogenesis and dendritogenesis, and the serotonergic system is impaired in DS. This impairment may thus contribute to the alteration of brain development. The group of Renata Barthesaghi found that in neonatally-treated Ts65Dn mice, one of the most widely used mouse model of DS, there the development of the hippocampus was rescued (a structure that in rodents mainly develops postnatally) in terms of neurogenesis, dendritogenesis and connectivity. Importantly, these effects largely outlasted treatment cessation and restored hippocampus-dependent memory (Clark *et al.*, 2006; Bianchi *et al.*, 2010b). They then wondered whether embryonic treatment with fluoxetine also rescued other aspects of brain development. They treated pregnant Ts65Dn females from embryonic day ten to delivery and examined the progeny at P2 and P45, and found that embryonic treatment with fluoxetine restored neurogenesis and cellularity throughout the whole brain of P2 Ts65Dn mice and that this effect was retained after treatment cessation and led to restoration of memory in P45 mice (Guidi *et al.*, 2014). The effects of treatment with fluoxetine in Ts65Dn mice were associated with restoration of the expression of 5-hydroxytryptamine 1A (5-HT1A) receptors and hippocampal serotonin levels suggesting that the serotonergic system may be involved in the beneficial effects of treatment. DS linked neurodevelopmental alterations were thought to be irreversible. This study demonstrated that it is possible, at least in the mouse model, to pharmacologically fully rescue brain development. Fluoxetine is an antidepressant that is also prescribed in children. However, its usage during pregnancy may not be free of side effects. Although the question is not completely settled, it appears

that fluoxetine during pregnancy may have effects on development of the heart (Reefhuis *et al.*, 2015), a process that is impaired in many children with DS.

Barthesaghi and coll. had previously obtained evidence that in trisomic neural precursor cells excessive levels of APP cause excessive levels of the APP Intra Cellular Domain (AICD) peptide, one of its cleavage products. AICD increases the transcription of *Ptch1*, the repressor of the mitogenic Sonic Hedgehog (Shh) pathway and that inhibition of this pathway is involved in neurogenesis impairment in DS (Giacomini *et al.*, 2015). Therefore, they thought that by reducing the levels of AICD could reinstate the functionality of the Shh pathway and, consequently neurogenesis. Since AICD derives from the cleavage of the APP proteolytic products carboxy terminal fragment (CTF) operated by gamma-secretase, in order to reduce AICD formation we used an inhibitor of gamma-secretase (ELND006). They found that neonatal treatment restored hippocampal neurogenesis and synaptic maturation (Giacomini *et al.*, 2015). This suggests that inhibitors of gamma-secretase may be used in order to restore brain development in DS. However, the inhibitor that we used caused adverse effects in a clinical trial in individuals with AD (Hopkins, 2011). Thus, their results provide proof-of principle demonstration of the usefulness of inhibitors of gamma-secretase in DS, but the transfer to humans requires the creation of safe inhibitors.

Glycogen synthase kinase 3 beta (GSK3-beta) is a kinase involved in many developmental processes, including neurogenesis and neuron differentiation (Jope & Johnson, 2004). Unlike other kinases, GSK3 becomes inactive when it is phosphorylated. Barthesaghi and coll. previously found that in trisomic neural precursor cells there are reduced levels of pGSK3-beta and that treatment with lithium, an inhibitor of GSK3beta activity, restored proliferation (Trazzi *et al.*, 2014). Based on these premises we decided to use *tideglusib*, a new selective non-ATP competitive inhibitor of GSK3-beta (Eldar-Finkelman & Martinez, 2011) in order to establish whether such a treatment may be useful to restore neurogenesis in DS. They found, however, that neonatal treatment with *tideglusib* had no

positive impact on neurogenesis. We tested a wide range of concentrations in cultures of neuronal precursor cells (NPCs) from Ts65Dn mice, but none was effective. These results are surprising because inhibition of GSK3-beta by lithium positively impacts neurogenesis. It must be noted that lithium has additional effects in the cell (Can *et al.*, 2014), suggesting that its efficacy on proliferation may not be directly linked to GSK3-beta inhibition. It is also possible that *tideglusib* has other cellular effects, not described so far, that counteract its effects on GSK3-beta. In any case, although *tideglusib* apparently improve behavior in mouse models of AD, it is not a suitable drug for DS.

Brain-Derived Neurotrophic Factor (BDNF) is a neurotrophin important for neurogenesis and neuron maturation. Since the DS brain exhibits reduced levels of BDNF, this defect may impair brain development and a treatment targeted to the BDNF system may have a beneficial effect. BDNF crosses the blood-brain barrier poorly, but this problem can be circumvented by using small molecules that bind to Tropomyosin receptor kinase B (TrkB). Based on these premises, the group of Renata Bartesaghi sought to establish whether treatment with 7,8-Dihydroxyflavone (7,8-DHF), a small flavonoid that binds with high specificity to TrkB (Liu *et al.*, 2010), positively impacts on neurogenesis and dendritic maturation. In Ts65Dn mice neonatally-treated with 7,8-DHF they found an increase in the proliferation rate of NPCs of the DG and an increase in spine density on the dendritic tree of granule neurons. These preliminary results show that pharmacotherapy targeted to the BDNF system positively impacts the two major defects of the trisomic brain. The duration of the effects of treatment remains to be established.

*DYRK1A* is a dual-specificity tyrosine phosphorylation-regulated kinase located on the HSA21 and is one of the triplicated genes strongly involved in DS phenotypes (for a recent review (Duchon & Herault, 2016)). Several studies in mouse transgenic models with either exogenous promoter constructs (TgDyrk1a) (Altafaj *et al.*, 2001), large genomic fragments in yeast artificial chromosomes (YACs) (Smith *et al.*, 1995; Smith & Rubin, 1997) or bacterial artificial chromosomes (BACs) (Ahn

*et al.*, 2006; Guedj *et al.*, 2012), showed that *DYRK1A* overexpression induces cognitive features of DS. Similarly, normalizing the overdosage of *Dyrk1A* in trisomic mouse models, Ts65Dn or Dp1Yey, reverts spatial working, reference memory and contextual conditioning deficits (Garcia-Cerro *et al.*, 2014) as well as T maze and fear conditioning deficits (Jiang *et al.*, 2015). In *Dyrk1A* overexpressing models, inhibition of DYRK1A kinase activity with EpiGalloCatechin Gallate (EGCG), a catechin of green tea extracts inhibitor of DYRK1A (Tejedor & Hammerle, 2011), restores many developmental and behavior defects (Guedj *et al.*, 2009; Pons-Espinal *et al.*, 2013; Thomazeau *et al.*, 2014). Importantly, the pioneer study by De la Torre, Dierssen *et al.* (De la Torre *et al.*, 2014) showed that adult TgDyrk1A and Ts65Dn mice treated with EGCG restore learning and memory deficits. Moreover, in a pilot study in young adults with DS, they showed that EGCG induces a behavioral benefit in some memory domains (De la Torre *et al.*, 2014). However, this benefit tended to disappear with time, when the treatment was discontinued. Based on this promising study, Barthesaghi and colleagues wondered whether treatment with EGCG during a crucial phase of brain development, when most of the hippocampal neurons are generated, rescues hippocampal architecture and whether the hippocampus may remain in its restored state after treatment cessation. They found that neonatal treatment with EGCG fully restores neurogenesis and synaptic development. Similar to the human study, at one month after treatment cessation, these effects were no longer present and there was no behavioral improvement (Stagni *et al.*, 2016). These results show that EGCG is a very good therapy for restoration of neurogenesis in DS, although its effects are ephemeral. Yet, since EGCG appears to be a safe compound, it may be possible to envisage a protocol of EGCG administration in which EGCG is periodically administered at time intervals –to be established– or in conjunction with other intervention (e.g. cognitive training) in order to prevent the disappearance of its effects. Specifically, the group of Dierssen demonstrated that the effects of EGCG were potentiated by enriched environment in DS mouse models, and thereafter, this was also shown in humans since combined treatment was more effective in human studies than EGCG alone (de la Torre *et al.*, 2016). Thus, it would be important to establish a concomitant environmental stimulation where administering

EGCG. Finally, de la Torre and Dierssen also showed pro-cognitive effects in *Fmr1* knockout mice (Dierssen *et al.* in preparation), thus already proving the possibility of using common treatment for DS and FXS.

New synthetic inhibitors of DYRK1A activity, with high purity and specificity, have been tested successfully showing recovery of cognition in DS mouse models (Gourdain *et al.*, 2013; Falke *et al.*, 2015; Kim *et al.*, 2016; Neumann *et al.*, 2018). Cognitive restoration with DYRK1A inhibitors was similar to the genetic rescue observed in adult DS mouse models when one functional copy of *Dyrk1A* was inactivated to bring back the *Dyrk1A* dosage to 2 copies (Ortiz-Abalia *et al.*, 2008; Garcia-Cerro *et al.*, 2014). In addition, Yann Herault and colleagues performed a quantitative phosphoproteomic approach that identified several specific protein targets of DYRK1A activity involved in synaptic function, leading to a better understanding of the pathophysiological alterations produced by DYRK1A overexpression (Y. Hérault *et al.*, in preparation). It will be important to find out whether similar molecular pathways are altered in various developmental disorders such as DS, FXS and 22q11.2 deletion.

The brief revision above already shows that the efforts of preclinical studies, may offer to clinicians a series of compounds that may be worthwhile testing in children with DS and possibly also with FXS. Since neurodevelopmental disorders share, sometimes, similar defects these compounds may also be exploited for other disorders. Given that they are effective, this achievement may offer a better life to affected children and their families.

### **Computational modeling and new approaches to link omics data with disease mechanisms**

In the last years, computational approaches have been successful to unravel the pathophysiology of many different diseases. Models of neural diseases link the biological and pathological abnormalities of the damaged brain, but to this aim computational neuroscience requires a battery of supporting



data obtained in the same experimental context and covering different levels of detail (neural types, neuromodulators, stimulus specific responses, etc.). Computational models help to capture the essential features of the brain at multiple spatio-temporal scales, from molecular networks and protein interaction to membrane currents and chemical coupling to network oscillations. Therefore, they offer a powerful tool to relate these data to neurological dysfunction. Moreover, models provide a theoretical framework where to control and manipulate neural networks under different “configurations” beyond the wet lab experiments, hence revealing unexpected results, otherwise counterintuitive or experimentally hard (or impossible) to obtain. In DS, due to the complexity of gene interactions and the outnumbered molecular pathways activated by the extra HSA21 copy, the interest in the over-expression of those genes that recapitulate the DS phenotype increased (Dierssen, 2012). On the other hand, research on DS patients is abundant and allows for model validation. However, despite the amount of experimental results available for data-driven models, DS has not driven the attention of computational neuroscience. According to the existing data on DS patients (and DS mouse models) covering network architecture (dendrite morphology) and macroscopic functional changes (brain activity alterations), we claim that both single-neuron-based and population networks can boost our understanding of DS. In addition, since the genetic causes of the disease are well known, computational models of DS can comprise another layer of biological description, i.e. the genetic regulatory network, that have remained so far detached from the modeling of neural circuits.

Another fundamental problem is that alterations of the nervous system leading to DS show a lifetime-specific development of neuropathological mechanisms that give raise to characteristic behavioral manifestations. Thus, we need to understand the progression of all the preclinical and clinical symptoms of such disorders, including patterns of behavior, what is commonly known as the natural history of the disorder, and the developmental and adult changes that produce specific cognitive

disturbances. To this aim, using novel bioinformatics approaches to understand big behavioral data will be essential in the search of treatments.

As an example, Faundez and coll. generated protein-protein interaction graphs between two key proteins triplicated in DS (DYRK1A and APP) and the protein absent in FXS (FMRP), and their interactors coming out from MS/MS analyses (Shannon *et al.*, 2003; Camargo *et al.*, 2007; Warde-Farley *et al.*, 2010; Wang *et al.*, 2011; Havugimana *et al.*, 2012; Corominas *et al.*, 2014; Huttlin *et al.*, 2015; Wan *et al.*, 2015). Fig. 1 shows either direct or indirect interactions between these three proteins highlighting hub proteins such as RBP9 (Ran-Binding Protein 9) a protein that binds RAN, a small GTP binding protein involved in nuclear translocation. It interacts with FMRP and modulates its RNA-binding properties (Menon *et al.*, 2004). In addition, RBP9 interacts with AICD inhibiting its transcriptional activity, and with APP decreasing A $\beta$  toxicity and cognitive deficits in mouse models of Alzheimer's disease (AD) (Domingues *et al.*, 2014; Woo *et al.*, 2015). Interestingly, DYRK1A inhibition improves AD pathology and cognitive deficits through inhibition of APP phosphorylation (Branca *et al.*, 2017). Validation of these interactions by testing pharmacological treatments in various mouse models across ID remains to be done.

In parallel, De Toma and coll. analyzed the overlap between FMRP1 targets (Darnell *et al.*, 2011) and a list of genes consistently deregulated in 45 studies on DS (Darnell *et al.*, 2011; Vilardell *et al.*, 2011; Toma *et al.*, 2016). Twenty-two genes were significantly shared ( $p < 4.5e-07$ , odds ratio = 3.78, Fisher test), 7 of which originating from brain studies ( $p < 0.0004$ , odds ratio 5.66, Fisher Test) (Table 2). Using *STRINGdb* (combined score  $>900$ ), they built the protein-protein interaction network of FMRP1 targets and DS genes (Fig. 2). They found 530 proteins involved in 1602 interactions, almost twice the number expected by chance (870). Interestingly, FMRP targets and DS genes were not forming two separate clusters, but 577 of these interactions involved a DS gene and a FMRP1 target. When calculating the average distance between nodes they found that 22 proteins which were FMRP1 targets and DS genes were also closer in the protein-protein interaction graph with an average distance of 17.14 while the mean value for all interactions was 128.65. Interestingly APP, one of the FMRP1

targets and a DS gene mapping to HSA21 came out as a hub in the network (since the probability to find a protein with higher interaction by chance was lower than 0.05).

Overall, the data from both analyses indicate that there are common molecular mechanisms shared across FXS and DS and that APP appears as an important target interacting with many important proteins involved in both diseases.

### **Bringing new avenues to modulate neuroplasticity in intellectual disabilities: the promising case of neurotherapy**

As indicated in this review there is a strong motivation to rescue the cognitive impairment in ID, including DS and FXS by researchers. Intellectual disabilities are associated with abnormal brain activity, which might be linked to immature development of connectivity between distant brain regions (Anderson *et al.*, 2013). Such delay in brain development affects the development of coherent distributed networks (Anderson *et al.*, 2013). This abnormal neuroplasticity might be affected by changes in excitation/inhibition, critical during development and the acquisition of cognitive skills (Cohen Kadosh *et al.*, 2015; Werker & Hensch, 2015).

Transcranial stimulation is a promising method to alter brain functions and modulate neuroplasticity. Both transcranial Direct Current Stimulation (tDCS) and transcranial Random Noise Stimulation (tRNS) showed long-term effects at the behavioural and neural level spanning from days to months after the intervention. Note that we do not review studies using Transcranial Magnetic Stimulation (TMS), another form of transcranial stimulation. While the potential use of TMS in combination with cognitive training/intervention remains to be explored, tDCS and tRNS, have higher practical validity for intervention, as they are portable, more comfortable for the participant, cheaper, easier to use in double-blind or sham-controlled studies, and more easily applied at the same time during training and for repeated use (Cohen Kadosh *et al.*, 2012; Krause & Cohen Kadosh, 2013). Moreover, when used within suggested guidelines, the acute safety risks (of seizures, for example) seem very low (in

contrast to TMS, there are no reports of seizures) with minimal discomfort or adverse side effects (Hummel & Cohen, 2006; Poreisz et al., 2007; Priori et al., 2009; Fertonani et al., 2015). The issue of safety is important as individuals with Down syndrome might be at increased risk of epileptic seizures.

### *tDCS*

tDCS involves the application of weak electrical currents, typically between 1-2mA through saline-soaked sponge electrodes from a battery-driven stimulator. It is the most common form of transcranial electrical stimulation used in studies on cognitive enhancement (Kuo & Nitsche, 2012; Coffman *et al.*, 2014; Looi *et al.*, 2016; Harty *et al.*, 2017) and neurointervention (Krause & Cohen Kadosh, 2013).

Mechanistically, tDCS operates on the basis of electrical polarity. Anodal tDCS typically facilitates neuronal firing, while cathodal tDCS inhibits neural firing (Bindman *et al.*, 1964; Nitsche & Paulus, 2000a; Nitsche & Paulus, 2000b; Bikson *et al.*, 2004a; Bikson *et al.*, 2004b). The effects of tDCS are long-lasting, spanning from weeks to months post-stimulation. This includes also improvement of high-level cognitive functions such as numerical abilities and executive functions, but also visuomotor abilities and language (e.g., (Floel et al., 2008; Reis et al., 2009; Cohen Kadosh et al., 2010; Looi et al., 2016). The effect of tDCS on behaviour might be mediated by alteration of concentrations of  $\gamma$ -aminobutyric acid (GABA) (Stagg et al., 2009; Clark et al., 2011; Kim et al., 2014) glutamate and glutamine (Clark et al., 2011), consistently with the idea that tDCS affects the excitability/inhibitory balance (Krause et al., 2013). The long-term effect of tDCS probably involves mechanisms with similar features to long-term synaptic plasticity (Stagg et al., 2011) including processes that rely on protein (Nitsche et al., 2009), protein synthesis (Gartside, 1968b; a), NMDA receptors known to support long-term potentiation (Islam et al., 1995; Nitsche et al., 2003) and long-

term depression, and mediation by polymorphisms in the brain-derived neurotrophic factor (BDNF) gene (Fritsch et al., 2010).

### *tRNS*

tRNS is a relatively novel form of transcranial stimulation that was investigated experimentally in 2008 (Terney et al., 2008). It involves the application of alternating currents (e.g., between  $-0.5$  to  $+0.5$ mA) at different frequencies to the scalp, typically between 0.1-640Hz or 100-640Hz, known to be safe for humans. Although it shares many similarities in the principles of its operation to tDCS, i.e., delivered to the scalp via electrodes that are attached to a stimulator and similar sham setup (by limiting the time of delivery enough to induce a ‘stimulation sensation’), this technique is preferred over tDCS for allowing better blinding (sham) conditions given its higher cutaneous perception threshold (Ambrus et al., 2010; Fertonani et al., 2015). tRNS also provides excitatory stimulation to the brain areas beneath the electrodes simultaneously (i.e., at the same time, on the same subject, rather than anodal and cathodal stimulation as in tDCS) as it is oscillatory current and hence, polarity-independent (Terney et al., 2008). Furthermore, in a perceptual learning study, it induced stronger behavioural effects than tDCS (Fertonani et al., 2011).

tRNS enhances neuronal excitability by increasing the activity of sodium ion channels (Terney et al., 2008; Fertonani et al., 2011) and stochastic resonance, whereby signal detection is enhanced when noise is introduced into the neural system (Miniussi et al., 2010; van der Groen & Wenderoth, 2016). tRNS affected cognitive functions improving skill acquisition, and mathematical and numerical abilities (Fertonani et al., 2011; Popescu et al., 2016), in some cases with effects lasting months after the end of the intervention (Cappelletti et al., 2013; Snowball et al., 2013; Cappelletti et al., 2015). While the mechanisms of both techniques warrant further investigation, tDCS and tRNS enhance various human motor and cognitive abilities (see (Paulus, 2011) and (Cohen Kadosh et al., 2015) for a collection of reviews). Given the current results, and the strong potential in long-term effect, the

potential for applying such method to improve intellectual abilities, such as DS, is appealing. However, the application of tDCS and tRNS to the developing brain is quite sparse, with some initial promising results in pilot studies on children with atypical development (Costanzo et al., 2016; Looi et al., 2017). While there is more benefit to gain compared to the risk in the case of intervention in atypical development than cognitive enhancement in typical development (Maslen et al., 2014a; Maslen et al., 2014b), future studies would need to carefully monitor the benefit as well as potential side effects of brain stimulation on the developing brain (Krause & Cohen Kadosh, 2013; Davis, 2014). In addition, studies on developing animals are important in order to assess potential side effect overseen when stimulation is applied to the child's brain, as currently the work on safety on animal models are based mainly on adults. Future studies would allow assessing the efficacy, safety, and also provide more causal evidence for the neural factors that are involved in intellectual disabilities, and therefore have both basic and translational impact.

### **Why translational research fails in intellectual disability?**

Advances in understanding molecular and synaptic mechanisms of ID in FXS and DS syndromes through animal models have led to targeted controlled trials with pharmacological agents designed to normalize these underlying mechanisms and find molecular targets to improve clinical outcomes. However, several clinical trials failed to demonstrate efficacy of these targeted treatments to improve surrogate behavioural/cognitive endpoints. One major obstacle to the demonstration of efficacy in human trials has been the lack of generally accepted test battery to assess improvement in function in individuals with intellectual disability. In the absence of a gold standard method to examine the therapeutic effects of a therapeutic intervention on cognition, researchers are pushed to test for efficacy in a variety of domains using a lab-specific panel of assessments. This approach inevitably leads to problems in comparing results across different labs, and even to translating significant findings from mouse models to human studies. Because the ultimate goal in these disorders is to

ameliorate intellectual disability, the validation of robust and sensitive neuropsychological measures for tracking treatment response is essential.

The recently developed National Institutes of Health Toolbox Cognitive Battery (NIH-TCB) for ID has potential for assessing important dimensions of cognition in persons with ID, and several tests may be useful for tracking response to intervention. However, more extensive psychometric studies, and evaluation of its sensitivity to developmental and treatment-related change, will determine the true utility of the battery as a set of outcome measures (Hessl *et al.*, 2016).

Even though those standardized test batteries are urgently needed, they may not allow addressing the broad inter individual variability in brain disorders leading to intellectual disability. We cannot consider patients with ID or even more specifically, with a defined syndrome, as a homogeneous group, since individual differences influence the relationships between genotype and the emerging phenotype. If we aspire at precision medicine, one extremely important issue is to understand that differences in the cognitive and functional evolution of each individual may stem from genetic variants (genetic polymorphisms, such as in the serotonin transporter and ApolipoproteinE genes), biochemical (A $\beta$ -peptides levels) and physiological (sleep disorders) differences, medical comorbidities (hypothyroidism, depression), sociodemographic characteristics (gender), or life style (diet). Those differences may also modulate the response to treatment and may lead to unresponsive, responsive patients or even to individual-specific adverse events, and will certainly require that the evaluation tools and medical endpoints be customized (McCabe & McCabe, 2013; Liogier d'Ardhuy *et al.*, 2015).

In the case of DS the group of de la Torre showed several examples of these factors. The first is that nearly all adults with DS show neuropathology of Alzheimer's disease (AD), including amyloid deposition by their fourth-fifth decade of life. In a clinical study in DS adults, they showed that higher

plasma A $\beta$ 42 levels were associated to a higher score in the Dementia Questionnaire for People with Learning Disabilities (DLD; formerly known as DMR) and impaired communication skills in the Adaptive Behavior Assessment System (ABAS-II; (Hoyo *et al.*, 2015). Specifically, the larger the A $\beta$ 42 concentrations the worse the performance of tasks detecting pre-dementia-states in young DS adults (Hoyo *et al.*, 2015).

Modifiable risk factors for cognitive impairment have also received attention, and there is a growing literature of metabolic risk factors for cognitive impairment and therapeutic management. One example is thyroid dysfunction. Overt hypothyroidism is a well-known reversible factor causing cognitive impairment including dementia (Moon, 2016). Hypothyroidism is the most common endocrine problem in DS, and approximately 10% of children and between 13% and 50% adults with DS have congenital or acquired thyroid disease being the incidence hypothyroidism high. De la Torre *et al.*, demonstrated that Serum Thyroid Stimulating Hormon (TSH) levels during hypothyroidism are inversely proportional to performance on word fluency and working memory tasks, and years of treatment with L-thyroxine are predictive of better performance in object recognition tests in the DS population (Xicota *et al.*, in preparation).

One of the challenges in cognitive neuroscience is to delineate the genetic determinants of inter individual variations. In the euploid population, polymorphisms involving changes in dopaminergic activity have consequences on cognition. Specifically, genetic variants of COMTVal158Met and VNTR-DAT1 polymorphisms contribute to prefrontal cortex-dependent cognition in healthy population. However, there are few data about how such genetic variants may influence the DS phenotype that is mostly explained by the overexpression of genes encoded by chromosome 21. In fact, the genetic background modulates the phenotypic consequences of genetic variants, and thus, polymorphisms may have differential consequences in DS. In DS, the groups of de la Torre observed that genotypes conferring higher dopamine availability as Met carriers and 10-repeat homozygotes



resulted in improved executive function tasks that require mental flexibility (Del Hoyo *et al.*, 2016). Met carriers also presented worse social skills and self-direction, along with higher social deterioration as measured by the Dementia Questionnaire for People with Intellectual Disabilities (DMR). This suggests that COMTVal158Met and VNTR-DAT1 polymorphisms interact with the trisomic genetic background to influence DS phenotypes.

Other factors may also confer individual variability to the DS population. For example, sleep quality. Individuals with DS are particularly vulnerable to sleep-related disturbances including, snoring, cough, choke and exhibiting signs of restlessness, unusual sleeping positions, excessive sweating and periods of sleep apnea. In a recent clinical trial (de la Torre *et al.*, 2016), poor sleep quality was associated to worse visual memory skills in DS and daily living functioning, and early conversion to AD using the Pittsburgh Sleep Quality Index (PSQI). Also, sleep apnea and snoring were associated to a worse adaptive behavior, while only snoring was associated to higher rates in the DMR and higher A $\beta$ 42/A $\beta$ 40 ratio in plasma (de la Torre *et al.*, 2016).

Variability at different levels may overlap in each individual with DS in different ways, and may end up producing convergent or divergent cognitive and functional outcomes, through myriads of differential interactions. For example, in some subjects with congenital hypothyroidism, the consequent cognitive impairment could be compensated if bearing genetic or environmental modifiers moderating APP metabolism. Instead, a trisomic subgroup with increased A $\beta$  concentrations, which at the same time suffer from obstructive sleep apnea or respiratory distress, has more deleterious consequences on cognitive decline. Besides, despite the similar neuropsychological and functional pattern in DS at a group level, it is likely that a particular level of performance is achieved by different developmental trajectories in each individual (D'Souza & Karmiloff-Smith, 2011).

In FXS, despite a common genetic etiology, there is a wide-ranging variability in its clinical presentation. The significant heterogeneity in behavioral and cognitive deficits observed among individuals with FXS is explained in part by variations in residual levels of FMRP. The latter is determined by mosaicism of the CGG expansion size, methylation levels, and X chromosome inactivation. Several studies in both females and males have correlated the severity of intellectual disability with FMR1 activity and FMRP levels (Loesch et al. 2004). However, their home environment also influences the cognitive outcomes are by in males, but not in females (Dyer-Friedman et al. 2002). In fact, the sensitivity and floor effects of cognitive testing methods in very low functioning individuals have limited the studies on the relationship between cognition and the molecular pathology of FXS. Only by developing new testing methods and/or algorithms for interpreting results from available cognitive measures, we will be able to establish the link with FMRP levels. Also, more quantitative methods capable of analyzing FMRP levels are required for understanding the relationship between molecular pathology and clinical phenotypes. Recent observations report a cross-reaction between the anti-FMRP monoclonal 7G1-1 and the RNA binding protein Caprin 1 (El et al. 2012), suggesting that anti-FMRP antibodies may not be as exclusive as previously thought. Other FMR1-related measures, such as DNA-methylation patterns and the characterization of additional genetic variants at the exome level, using next generation sequencing tools, may provide other means of explaining phenotypic heterogeneity in FXS.

Due to the described variability, DS and FXS individuals do not show similar response to the same pharmacological treatment, which is an important consideration for establishing drug efficacy in clinical trials. We need to redefine primary end-points to assess improvement in function in individuals with ID and consider the factors, which explain part of the phenotype variability. Those would possibly configure a set of biomarkers assessed in the screening visit in the context of a drug-efficacy clinical trial for cognitive, behavioral and/or functional enhancement.



## Conclusions

Current therapeutic approaches for DS and FXS focus on behavioral therapy, educational mainstreaming and off-label medications that mitigate only a limited set of symptoms but no approved pharmacological therapies are yet available. The incomplete understanding of individual phenotypic variability, natural history, and causes of differential response to inform trial design have limited our capacity to succeed, even when very promising drugs are tested, and are possibly dismissing good therapeutic opportunities. This underlines the requirement for more basic research, new tools and models that will allow a better understanding of the pathophysiology of those syndromes. In recent years, the use of iPS cells reprogrammed from patient fibroblasts and further derived into neuronal cells and organoids, has boosted research on mechanisms involved in genetic developmental disorders leading to intellectual disabilities. Some examples come from the field of DS and FXS. Using these cellular models as well as validated mouse models, genomic, proteomic and interactome studies has led to the discovery of common pathways across diseases that can now be targeted for clinical studies. On the other extreme, clinical research has somehow been disappointing likely because of the heterogeneity inside specific intellectual disabilities of genetic origins taking place during development. Commonalities and grounds for stratification in this heterogeneous landscape still need to be defined. Nevertheless, it is also necessary to create more sensitive and realistic outcome measures to quantify disease and therapeutic efficacy, for improving patient recruitment strategies and access to resources required to mount a clinical trial (including funding). Solutions will require multicenter collaboration, partnership with patient organizations, training new generations of researchers and increasing the public resources dedicated to this field. More fundamental, applied and clinical research will need to be performed and various domains scrutinized for possible applications in various IDs.

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## Figure legends

Fig. 1. The FMRP and DYRK1A interactome.

Diagram depicts the putative interactome linking FMRP1 and DYRK1A. Note that APP acts as a hub connecting FMRP and DYRK1A. The interactome was obtained from six published proteome-wide interactomes datasets (Shannon *et al.*, 2003; Camargo *et al.*, 2007; Warde-Farley *et al.*, 2010; Wang *et al.*, 2011; Havugimana *et al.*, 2012; Corominas *et al.*, 2014; Huttlin *et al.*, 2015; Wan *et al.*, 2015). These six datasets were analyzed and curated using the Genemania platform (Warde-Farley *et al.*, 2010). Binary interactions obtained in Genemania were plotted using Cytoscape 3.5.1 (Shannon *et al.*, 2003).

Fig. 2: FMRP1 targets and DS genes interactions.

Protein-protein network interaction of FMRP1 targets (blue nodes) and DS genes (red nodes). 22 proteins were both DS genes and FMRP1 targets (black nodes). Proteins in the top 5% right tail of the interaction distribution—so called “hubs”—are indicated with bigger size. Edges between two DS genes are in red, between two FMRP1 targets in blue, and edges connecting both FMRP1 targets and DS genes in black. DS genes found in brain studies are represented by squares instead of circles. The distance of an unconnected node was considered as the total number of nodes -1.

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